DNA Constructs

The present invention concerns novel DNA constructs, host cells comprising said constructs, methods and processes for the production of a desired protein, particularly a therapeutically useful protein, DNA vaccines and other medicaments comprising said constructs and methods of gene therapy using said constructs.

Eukaryotic Initiation Factor 4A (eIF-4A) is a 43kD protein with RNA helicase activity that mediates binding of mRNA to the 40S subunit of the ribosome. In the mouse genome there are two functional eIF4A genes, eIF4AI and eIF4AII (1,2). The two eIF4A genes are differentially regulated in the mouse with $eIF4A_I$ mRNA being expressed at higher levels than the $eIF4A_{II}$ mRNA in dividing cells with the $eIF4A_{II}$ mRNA being preferentially expressed in non dividing cells (3). In the human genome the homologous eIF-4AI and eIF-4AII genes have been mapped to chromosomes 17p13 and 18p11.2 respectively (4,5).

In common with many other 'housekeeping genes' the gene encoding eIF-4AI must be expressed in every mammalian cell type at approximately similar levels. Many housekeeping gene promoters do not contain TATA box sequences to direct transcription initiation but rather have GC rich promoters that contain binding sites for the transcription factor Sp1 and initiator elements (6). Many housekeeping gene promoters contain a higher than expected frequency of the dinucleotide CpG and these regions are not as extensively methylated as other CpG dinucleotides elsewhere in the genome (7). Such Methylation Free Islands (MFI) are often associated with transcriptional regulatory regions of genes and are thought to act by maintaining adjacent chromatin regions in an open configuration (8).

Cloning of the genes defective in human monogenic diseases such as the Cystic Fibrosis has raised the possibility of introducing a non mutated form of the Cystic Fibrosis Transmembrane Regulator (CFTR) gene into the appropriate cell types of affected individuals to restore normal function. In order for such somatic gene therapy protocols to be useful the therapeutic gene of interest must be stably expressed at a level similar to or higher than that of the mutated gene. Nearly

all somatic gene therapy vectors used to date have relied upon transriptional regulatory regions derived from viruses such as the SV40 enhancer, retroviral Long Terminal Repeat sequences or the promoter/enhancer element of the human Cytomegalovirus immediate early gene. While recombinant retroviruses can efficiently infect dividing primary cells gene expression from integrated proviral genomes is often unstable (9). Similar problems have been encountered with long term gene expression using episomal viral vectors (10).

More recently *in vivo* delivery of plasmid DNA containing antigen genes under the control of viral promoters has been used to elicit humoral and cell mediated immune responses in experimental animal models (11). It is not yet clear what properties the ideal mammalian expression vector for DNA vaccination should have in order to elicit an effective immune response. Many currently used viral promoters do not direct high levels of gene expression in professional antigen presenting cells. Thus a promoter which can drive expression in macrophages and other cell lines where CMV gives poor expression, e.g. prostate cancer cells, would be desirable for both genetic vaccination and gene therapy.

It has also been found that viral promoters are rapidly silenced and thus fail to give more than transient expression of therapeutic or prophylactic genes. Thus there is a need for a ubiquitously active promoter which can be used as a universal promoter for all types of gene therapy.

It is therefore an object of the present invention to provide alternatives to the currently used viral promoters.

In accordance with the present invention there is provided a DNA construct comprising a transcriptional regulatory sequence operatively linked to a heterologous gene of interest wherein the transcriptional regulatory sequence comprises a transcriptional regulatory polynucleotide which is the eIF4A gene promoter, a fragment thereof or a polynucleotide hybridisable thereto.

In accordance with a preferred aspect of the present invention the construct comprises at least one Eif4a gene intron, fragment thereof or polynucleotide hybridisable thereto.

In accordance with a more preferred aspect of the present invention the eIF4a gene intron is intron 1, 2, 3, 5, 6, 7 or 9.

In accordance with a further aspect, there is provided a host cell comprising a DNA construct as hereinbefore described.

In accordance with a further aspect, there is provided a process for the production of a protein of interest, which process comprises the step of culturing said cells as hereinbefore described.

In accordance with another aspect, there is provided a DNA vaccine comprising the construct as hereinbefore described. Methods for the treatment, including prophylatically where appropriate, of diseases or disorders comprising the step of administrating said vaccine are also provided. Gene therapy methods comprising the step of administrating said construct are also provided.

The present invention provides DNA constructs that are capable of expressing a gene of interest over a longer time period than provided for by the viral promoters such as the human CMV promoter. Such expression characteristics are particularly beneficial where long term expression is required such as in gene therapy, vaccination treatments and in the commercial production of proteins.

The heterologous gene of interest is a gene which does not code for eIF4a. Typically the heterologous gene of interest encodes a protein of interest.

In preferred embodiments, the construct of the present invention is non-chromosomal e.g. a phage, plasmid, virus, minichromosome or transposon. Of these, plasmid is particularly preferred.

SEQ.I.D.NO:38 sets forth the sequence of the human eIF4A1 promoter from position -526.

By the term "derivable" it is intended to convey a source not only in the sense of it being the physical source for the material but also to define material which has structural and/or functional characteristics which correspond to those materials but which does not originate from the reference source. The polynucleotide is preferably derived from a mammalian source, particularly murine, rattus or human, preferably human.

Fragments of the eIF4AI promoter may be used in place of, or in addition to the full length promoter sequence so long as they retain the biological characteristic of a promoter when incorporated into constructs of the present invention. Indeed, the present inventors have demonstrated that such fragments may be obtained from the full length eIF4AI promoter sequence. Accordingly, such fragments include -526EIF, -371EIF, -271EIF, -193EIF, -120EIF, -98EIF, -69EIF and -40EIF.

Variants of the wild type eIF4AI promoter sequence are also encompassed within the present invention. Such variants may be naturally occurring variants which may have a substitution, deletion or insertion of one or more bases. Variants also include non-naturally occurring variants in which one or more bases have been added, substituted, inserted, deleted, rearranged or modified yet retain promoter characteristics. Also encompassed within the scope of the present invention are variants having at least 90% identity with the eIF4AI promoter or fragment thereof, e.g. 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or even 99% identity.

Polynucleotide molecules which hybridise to the eIF4A gene promoter, or to complementary nucleotides thereto also form part of the invention, provided that they retain the transcriptional regulatory function of a promoter. Similarly polynucleotide molecules which hybridise to at least one eIF4A gene intron, or to complementary nucleotides thereto also form part of the invention, provided that they retain the transcriptional regulatory function. Hybridisation is preferably under stringent hybridisation conditions. One example of stringent hybridisation conditions is where attempted hybridisation is carried out at a temperature of from about 35°C to about 65°C using a salt solution which is about 0.9 molar. However, the skilled person will be able to vary such conditions as appropriate in

order to take into account variables such as probe length, base composition, type of ions present, etc.

In preferred forms, the transcriptional regulatory sequence comprises least one eIF4A intron, fragment thereof or polynucleotide hybridisable thereto. In particular, it is preferred that the intron is derivable from intron 1, 2, 3, 5, 6, 7 or 9. A fragment of the intron will retain the transcriptional regulatory function of the intron and is typically at least 15, 20, 50 or 100 nucleotides long. Of these it is particularly preferred that the polynucleotide is derived from intron 1 of the eIF4AI gene. It is preferred that the intron polynucleotide is derived from a mammalian source, e.g. murine, rattus or human, preferably human.

Variants of the intron sequence in which one or more bases have been added, substituted, inserted, deleted, rearranged or modified yet retain enhancer characteristics are also encompassed within the invention. Variants of the transcriptional regulatory sequence having at least 90% identity, e.g. 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or even 99% are also provided.

The present inventors have demonstrated advantage in utilising more than one intron derivable from eIF4AI gene. Thus, constructs may comprise a transcriptional regulatory sequence comprising a polynucleotide derivable from the eIF4A promoter or fragment thereof and a plurality of eIF4AI gene introns. The additional intron sequence may be the same or different from the preceding intron. It is preferred that the polynucleotide comprises the eIF4AI promoter sequence or fragment thereof and eIF4AI intron sequence or fragment thereof arranged in tandem in forward or reverse orientation. The intron sequence and promoter sequence may be co-terminus or spaced apart. The intron sequence may be upstream or more preferably downstream (in terms of reading frame) of the eIF4A promoter sequence. Chimaeric constructs e.g. one in which the polynucleotide comprises the eIF4A promoter or fragment thereof derived from a first species and the intron sequence derived from a second species are also envisaged. Preferably, the promoter sequence is produced by recombinant means, such techniques being standard and well known to those skilled in the art.

Constructs of the present invention may further comprise other elements such as a terminator sequence and a selectable marker, bacterial origin of replication, antibiotic resistance gene and a signal peptide gene for secretion if necessary. Constructs of the present invention are preferably incorporated into a host cell for gene expression although cell-free translation systems are not excluded. The incorporation of the constructs may be achieved by methods well known to those skilled in the art, e.g. P1 transduction. Suitable host cells maybe prokaryotic or eukaryotic, preferably eukaryotic, even more preferably mammalian. Host cells may be fully differentiated, pluripotent stem cells or other precursor cells. The present invention is particularly advantageous in directing high level persistent expression of a gene of interest in professional antigen presenting cells such as macrophages and dendritic cells and other cells that have been shown to be refractory to high level, persistent gene expression by viral promoters e.g. prostate and colorectal cancer cells.

Host cells transformed by constructs of the present invention may be used in the commercial production of proteins. Generally, a fermentation method is employed which involves submerging the cells in a culture medium contained within a suitable vessel. Following culturing at appropriate conditions, proteins secreted by host cells may be recovered from the culture medium by standard techniques known to those skilled in the art. It is preferred that the protein is recovered to homogeneity. In addition transformed host cells of the present invention maybe utilised in therapeutic strategies for replacing or compensating for cell loss. For example, mammalian host cells for transplantation (which maybe autologous or allogenic or less preferred xenogeneic) maybe transformed *ex vivo* with a gene construct of the present invention in which the gene of interest encodes dopamine for the treatment of Parkinson's disease although other cell loss diseases or disorders which may benefit from the present invention will be readily apparent to those skilled in the art. The host cells of the invention may be used to treat diseases characterised by clinically significant cell loss particularly neuronal cell loss. Viruses, particularly retroviruses and adenoviruses, incorporating the transcriptional regulatory sequence of the present invention are also envisaged. Embodiments in which the gene of interest encodes a prodrug converting enzyme or a suicide gene are also provided.

Of particular interest is the use of the constructs of the present invention in DNA vaccines and gene therapy. Examples of DNA vaccines that may benefit from the present invention include tumour vaccines, hepatitis B and C, HIV, tuberculosis, HPV and HSV vaccines and vaccines directed at modifying chronic inflammatory reactions, such as MS, asthma, RA and Alzheimers, or directed at other biological pathways, such as vaccines for contraception or drug addiction.

The inventors have further found that the transcriptional regulatory sequence of the invention can be used to bias the immune response provoked by a DNA vaccine. Typically the immune response is biased towards Th1 and away from Th2. The present invention therefore provides a method of treatment which method comprises administering to a patient an affective amount of a construct or vaccine according to the invention and obtaining a biased immune response. The invention further provides the use of a construct as defined herein for obtaining an immune response which is biased to Th1.

Constructs may be administered in conjunction with a carrier such as, for example, liposomes. Typically such liposomes are cationic, for example imidazolium derivatives (WO95/14380), guanidine derivatives (WO95/14381), phosphatidyl choline derivatives (WO95/35301), piperazine derivatives (WO95/14651) and biguanide derivatives. The construct may comprise a gene of interest such as CTFR or erythropoetin gene operatively linked to the promoter sequence. Thus a method of correcting or compensating for a disease or disorder whose etiology is characterised by a genetic aberration (such as cystic fibrosis) is provided, which method comprises the step of administrating to a mammalian patient in clinical need thereof a therapeutically effective amount of the construct, preferably incorporated into a carrier. In a particularly advantageous use, constructs of the present invention may be used in driving high level persistent gene expression in avelolar macrophages.

The promoter sequence of the present invention is operatively linked to a gene of interest. Genes of interest include genes encoding for therapeutic or non-therapeutic proteins. Therapeutic proteins include those whose large scale commercial production is hampered by the prohibitive cost of current techniques.

The heterologous gene of interest may encode a protein which is not normally expressed in the host cell. By way of illustration, genes encoding for proteins such as insulin, sTNFr, interferon-β, factor VIII, erythropoetin, growth factors and cytokines or therapeutically effective fragments thereof, are all examples of genes of interest. For example the construct of the invention may be used in gene therapy to achieve over-expression of human genes such as VEGF for induction of angiogenesis.

Medicaments, particularly pharmaceutical compositions, comprising the construct of the invention, the host cell of the invention or therapeutic proteins produced according to the present invention are also provided. The elf4A promoter is active in all cell types, i.e. it is a ubiquitously active promoter, and gives sustained high level expression in all cell types.

In accordance with further aspects of the present invention, isolated forms of introns 1 (SEQ.I.D.NO:31),2 (SEQ.I.D.NO:32),3 (SEQ.I.D.NO:33),5 (SEQ.I.D.NO:34),6 (SEQ.I.D.NO:35),7 (SEQ.I.D.NO: 36) and 9 (SEQ.I.D.NO:37) are provided. By the term "isolated" we mean that the nucleic acid as described herein exists in a physical milieu distinct from that in which it occurs in nature. For example, the nucleic acid may be isolated with respect to one or more materials it is associated with in the natural state. Thus in accordance with the present invention, there is provided an isolated polynucleotide comprising or consisting essentially of the sequence as set forth in SEQ.I.D.NO:31 or 32 or 33 or 34 or 35 or 36 or 37 or variant thereof, fragment thereof or polynucleotide hybridisable thereto. A recombinant DNA construct comprising a transcriptional regulatory sequence which regulatory sequence comprises an isolated polynucleotide as described herein is also provided. Isolated polynucleotides as identified herein may be used as enhancer elements in transcriptional regulatory sequences found in recombinant DNA constructs according to the invention. In other aspects, a recombinant DNA construct comprising an transcriptional regulatory sequence operatively linked to a heterologous gene of interest which regulatory sequence comprises or consists essentially of a polynucleotide having a sequence as set forth in SEQ.I.D.NO:38 or variant thereof preferably further comprising a sequence as set forth in any one of SEQ.I.D.NO:31, 32,33,34,35,36,37 or variant thereof is provided.

In other aspects there is provided an isolated polynucleotide having a sequence as set forth in SEQ.I.D.NO:40 at positions -2102 to -1082 or variant thereof.

There is also provided an isolated polynucleotide having a sequence as set forth in SEQ.I.D.NO:40 at positions -1107 and -505 or variant thereof.

Suitable techniques for introducing the naked polynucleotide or vector into a patient include topical application with an appropriate vehicle. The naked polynucleotide or vector may be present together with a pharmaceutically acceptable excipient, such as phosphate buffered saline (PBS). One technique involves particle bombardment (which is also known as 'gene gun' technology or Particle Mediated DNA Delivery and is described in US Patent No. 5371015). Here inert particles (such as gold beads) are coated with a nucleic acid, and are accelerated at speeds sufficient to enable them to penetrate a surface of a recipient (e.g. skin), for example by means of discharge under high pressure from a projecting device. (Particles coated with a nucleic acid molecule of the present invention are within the scope of the present invention, as are devices loaded with such particles.) Other methods of administering the nucleic acid directly to a recipient include ultrasound, electrical stimulation, electroporation and microseeding which is described in US-5,697,901.

A nucleic acid sequence of the present invention may also be administered by means of specialised delivery vectors useful in gene therapy. Gene therapy approaches are discussed for example by Verme *et al*, Nature 1997, **389**:239-242. Both viral and non-viral systems can be used. Viral based systems include retroviral, lentiviral, adenoviral, adeno-associated viral, herpes viral and vaccinia-viral based systems. Non-viral based systems include direct administration of nucleic acids and liposome-based systems.

The constructs of the invention may be administered as pharmaceutical compositions, which are typically administered in any effective, convenient manner including, for instance, administration by parenteral or other routes, for example topical, oral, anal, vaginal, intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal or intradermal routes among others.

The pharmaceutical compositions generally are administered in an amount effective for treatment or prophylaxis of a specific indication or indications. In general, the compositions are administered in an amount of at least about 1 µg/kg body weight. In most cases they will be administered in an amount not in excess of about 8 mg/kg body weight per day. Preferably, in most cases, dose is from about 1 µg/kg to about 1 mg/kg body weight, daily. It will be appreciated that optimum dosage will be determined by standard methods for each treatment modality and indication, taking into account the indication, its severity, route of administration, complicating conditions and the like. For DNA vaccines, the dosage will typically be 1 – 10 µg per administration, for example 1 – 10 µg prime, followed by at least one boost of 1 – 10 µg, administered at 1, 2, 3 or 4 week intervals.

In therapy or as a prophylactic, the construct may be administered to an individual as an injectable composition, for example as a sterile aqueous dispersion, preferably isotonic.

Alternatively the composition may be formulated for topical application for example in the form of ointments, creams, lotions, eye ointments, eye drops, ear drops, mouthwash, impregnated dressings and sutures and aerosols, and may contain appropriate conventional additives, including, for example, preservatives, solvents to assist drug penetration, and emollients in ointments and creams. Such topical formulations may also contain compatible conventional carriers, for example cream or ointment bases, and ethanol or oleyl alcohol for lotions. Such carriers may constitute from about 1% to about 98% by weight of the formulation; more usually they will constitute up to about 80% by weight of the formulation.

For administration to mammals, and particularly humans, it is expected that the daily dosage level of the active agent will be from 1 µg/kg to 10 mg/kg, typically around 1 mg/kg. The physician in any event will determine the actual dosage which will be most suitable for an individual and will vary with the age, weight and response of the particular individual. The above dosages are exemplary of the average case. There can, of course, be individual instances where higher or

lower dosage ranges are merited, and such are within the scope of this invention.

Brief description of drawings and sequences

SEQ.I.D.NO:39 DNA sequence of the promoter and exon 1 of the human eIF4A1 gene. DNA sequences are numbered with the putative transcription site denoted as +1. Potential transcription factor binding sites are underlined. The amino acids encoded by exon 1 are shown. eIF4A1 gene sequence was determined by DNA sequencing of the cosmid clone cosCD68C1 (Jones, E., Quinn, C.M., See, C.G., Montgomery, D.S., Ford, M.J., Kolble, K., Gordon, S., and Greaves, D.R. (1998), Genomics 53: 248-250).

SEQ.I.D.NO:40 DNA sequence of 5.318 kb upstream of the human eIF4A gene. This sequence starts from the 5' Not I site of cosmid cos CD68C1, at –5280, where the transcriptional start site of the human eIF4A gene is +1, (SEQ.I.D.NO:39). It also includes the promoter and exon 1 sequence shown in SEQ.I.D.NO:39 and ends at the final base of exon 1, (+37). Regions of homology to Alu I repeat sequences or to the mouse eIF4A gene and surrounding sequences are underlined,(see Figure 1). The regions cloned and assayed for transcriptional actvity as PCR A (-2102 to –1082) and PCR B (-1107 to –505) are highlighted in bold or italics, respectively, (see Figures 1 and 3).

Fig.1: Illustrates the organisation of the human eIF4A1 gene. Coding sequence exons of the eIF4A1 and CD68 genes are represented by black boxes, introns by white boxes and 3' untranslated regions by shaded boxes. The position of Alu I repeat sequences is shown by horizontal arrows below the line. Vertical lines with arrowheads denote the transcription start sites of the two genes, poly A addition sites are represented by the symbol pA^+. Hatched boxes delineate the regions of the human eIF4A1 gene sequence which share homology with the murine eIF4A1 promoter and the position of the CpG rich methylation free island within intron 1. White and black boxes represent the positions of promoter fragments, (PCR A and PCR B respectively), assayed for transcriptional activity in fig.3.

Fig.2: Mapping the transcriptional regulatory sequences of the eIF4A1 promoter. The indicated luciferase reporter plasmids were introduced into CHO, 293 and RAW264.7 cells along with a beta-galactosidase reporter gene plasmid (pcDNA Beta gal) as described below. Cell lysates were prepared 16 hours after transfection and assayed for luciferase and beta galactosidase enzyme activities. Cell lysates were diluted in cell lysis buffer to ensure that all luciferase assays were within the linear range of the enzyme assay. The error bars represent the standard error of the mean of two independent transfection experiments.

Fig.3: Transcriptional regulatory sequences in eIF4A1 5' flanking sequence. Plasmid DNAs of the indicated plasmids (20μg) were introduced into RAW264.7 cells by electroporation along with plasmid DNA (2μg) for a beta galactosidase expression vector (pcDNA3 Beta gal). A and A', B and B' represent forward and reverse orientations respectively of the PCR fragments shown in fig.1. Cell lysates were prepared 16 hours after transfection and assayed for luciferase and beta galactosidase enzyme activities. Luciferase enzyme activities are expressed relative to the luciferase activity obtained with the plasmid -40eIF luc transfected in the same experiment. Similar results were obtained in two independent experiments.

Fig.4: eIF4A1 directs high-level expression in macrophage cell lines. Panel A. Plasmid DNAs of the indicated plasmids (20μg) were introduced into RAW264.7 cells by electroporation along with plasmid DNA (2μg) for a beta galactosidase expression vector (pcDNA3 Beta-gal). Cell lysates were prepared 24 hours after transfection and assayed for luciferase and beta-galactosidase enzyme activities. Error bars represent the standard error of the means of two independent experiments.

Panel B. RAW cells were electroporated with the plasmid hCMVluc or –271eIF-IVS1 and the co-reporter plasmid pcDNA3 Beta-gal. Cells were harvested at 16, 24, 36, 48, 72 and 96 hours post transfection and cell lysates assayed for luciferase activity. Relative luciferase activities are given as a percentage of the value obtained at 16 hours normalised for beta-galactosidase activity at 16 hours. Similar results were obtained in two independent experiments.

Panel C. CHO cells were transfected with the plasmid hCMVluc or –271eIF-IVS1 and the co-reporter plasmid pcDNA3 Beta-gal. Cells were harvested at 16, 24, 40 and 48 hours post transfection and cell lysates assayed for luciferase activity. Relative luciferase activities are given as a percentage of the value obtained at 16 hours normalised for beta-galactosidase activity at 16 hours. Similar results were obtained in two independent experiments.

Fig.5: The plasmid –40 IVS1 (x1) contains one copy of eIF4A1 gene intron 1 cloned downstream of the –40 eIF4A1 promoter fragment in pGL3Basic and plasmid –40 IVS1 (x2) contains two copies of eIF4A1 gene intron 1 cloned downstream of the –40 eIF4A1 promoter. The plasmid pGL3Control contains the SV40 promoter and enhancer sequences cloned into the luciferase reporter plasmid pGL3Basic.

The indicated plasmid DNAs were introduced into the murine macrophage cell line RAW 264.7, human 293 cells and Chinese Hamster Ovary (CHO) cells along with a beta-galactosidase co-reporter plasmid (pcDNA3 Beta-gal). Cells were harvested 16 hours post transfection and cell lysates assayed for luciferase and beta-galactosidase enzyme activities. Normalised luciferase activities are expressed as fold induction compared to the promoterless vector pGL3Basic.

Fig.6: The eIF4A promoter and intron 1 are active in cancer cell lines. Luciferase reporter plasmids were introduced into (A) LNCaP (human prostate cancer) and (B) Cos-1 (African green monkey kidney), cells by electroporation and into (C) WiDr (human colorectal cancer) and (D) SKOV-3 (human ovarian cancer) using lipofectin. Cell lysates were prepared 48 hours post transfection and luciferase assays were performed and standardized by total protein content. All experiments were repeated in triplicate and showed similar values, but in each case a representative single experiment is shown.
(HSV1Tkmin = minimal thymidine kinase promoter from Herpes Simplex Virus 1).

Fig.7: The eIF4A promoter and intron 1 direct gene expression in vivo in mouse muscle. Luciferase reporter plasmids (50μg) were injected into the quadriceps muscle of C57Bl.6 mice. Muscles were removed after 72 hours and assayed for luciferase activity and the values standardized by protein content. Data is the mean of five animals. (-40EIF IVS1 A = one copy of intron 1, -40eIF4A IVS1 B = two copies of intron 1).

Fig.8: Comparative luciferase activity of eIF4A and CMV promoter expression plasmids upon transient transfection into MK cells.

Fig.9: Time course of promoter activity in MK cells.

Fig.10: Efficacy of CMV and -271Eif-IVS1 promoters in particle mediated DNA delivery. Serum IgG response (1000 times dilution).

Fig.11: Efficacy of CMV and -271eIF-IVS1 promoters in particle mediated DNA delivery. Serum IgG response 2 weeks post boost.

Fig.12: CD8+ T cell response to pVAC1.PR construct with –271eIF4a promoter as shown by interferon-gamma ELISPOT assay of splenocytes collected 6 weeks post boost immunisation.

Fig.13: CD8+ T cell response to pVAC1.PR with -271eIF4a promoter as shown by Europium release CTL assay of splencytes collected 6 weeks after boost immunisation.

The present invention will now be illustrated by way of example only. It should be understood that these represent preferred embodiments of the invention. Various modifications and changes within the spirit and scope of the invention will be apparent to those skilled in the art.

Examples

DNA sequence of the human eIF-4AI gene. Restriction enzyme fragments of the recombinant cosmid cosCD68C1 containing the human eIF4AI gene were subcloned into pBluescript SK-. DNA sequence was determined on both

strands of the plasmid and cosmid templates using a Dye Terminator sequencing kit (ABI Perkin Elmer).

Reporter gene plasmids. Fragments of the human eIF4A1 promoter were PCR amplified to generate a 5' promoter deletion series using cosmid cosCD68C1 as template. Forward PCR primers included a Kpn I site and the reverse primer included a Hind III site, PCR products were digested with Kpn I and Hind III and cloned into the multiple cloning site of the luciferase reporter vector pGL3 Basic (Promega). Forward primers used were:

eIF–526 5' ATCTGGTACCCTACGATATCGCTGTTGATTTC (SEQ.I.D.NO:1),
eIF-371 5' ATCTGGTACCTGGAGGCTGAGACCTCGCC, (SEQ.I.D.NO:2)
eIF-271 5' ATCTGGTACCATGGCTGCCAGGCCTCGAGG, (SEQ.I.D.NO:3)
eIF-193 5' ATCTGGTACCGGCTGCGGGGCGGGCC, (SEQ.I.D.NO:4)
eIF-120 5' ATCTGGTACCTAGGAACTAACGTCATGCCG, (SEQ.I.D.NO:5)
eIF-98 5' ATCTGGTACCGTTGCTGAGCGCCGGCAGGC, (SEQ.I.D.NO:6)
eIF-69 5' ATCTGGTACCAAACCAATGCGATGGCCGG (SEQ.I.D.NO:7) and
eIF-40 5' ATCTGGTACCCGGGCGCTCTATAAGTTGTCG (SEQ.I.D.NO:8).

The reverse primer used was

5' ATATAAGCTTTGATCCTTAGAAACTAGGGC (SEQ.I.D.NO:9).

Regions of the human eIF4AI 5' flanking region which show homology to the mouse eIF-4AI 5' flanking sequence were PCR amplified using the primers
eIFBF 5' ATCTGGTACCGACTGGATTTCCACCAG (SEQ.I.D.NO:10) and
eIFBR 5' ATCTGGTACCACCCAGGGCCACAGG (SEQ.I.D.NO:11).

The region of non-homology with the murine eIF-4AI 5' flanking region was PCR amplified using the primers

eIFAF 5' ATCTGGTACCTGTGGCCCTGGGTGG (SEQ.I.D.NO:12) and
eIFAR 5' ATCTGGTACCGGAAATCAACAGCGATATCGT (SEQ.I.D.NO:13).

PCR products were digested with Kpn I and cloned in both orientations into the Kpn I site of eIF-40luc to give the plasmids BeIF-40luc, B'eIF-40luc, AeIF-40luc and A'eIF-40luc.

Introns of the eIF4A1 gene were PCR amplified with the following primers:

IVS1 F 5' ATCTAAGCTTCCCGGTAAGAAAGGCATTTG (SEQ.I.D.NO:13),
IVS1R 5' ATCTAAGCTTGGATCTGTTGGTTTAAAGCAT, (SEQ.I.D.NO:14)
IVS2F 5' ATCTAAGCTTGTCGACCCCGAAGGCGTCATCGAGGTGA
(SEQ.I.D.NO:15),
IVS2R 5' ATCTAAGCTTGAATTCTAGGGGATGCAAAGA (SEQ.I.D.NO:16),
IVS3F 5' ATCTAAGCTTGTATCAAGGGTGAGACC (SEQ.I.D.NO:17),
IVS3R 5' ATCTAAGCTTCATAACCTAAACAAATAAATT (SEQ.I.D.NO:18),
IVS4F 5' ATCTAAGCTTCTCAGCAGGTAAGAGTGG (SEQ.I.D.NO:19) ,
IVS4R 5' ATCTAAGCTTGAATTCCCTTCTGTATCTGAGCAG (SEQ.I.D.NO:20) ,
IVS5F 5' ATCTAAGCTTTGCTGGTTTCTCTCTGG (SEQ.I.D.NO:21),
IVS5R 5' ATCTAAGCTTGAATTCGGGCTAGAGAAGAAAAA (SEQ.I.D.NO:22),
IVS6F 5' ATCTAAGCTTCCCAGGTGAGGGCAGT (SEQ.I.D.NO:23) ,
IVS6R 5' ATCTAAGCTTGAATTCAGCAAAACTACCTAGTGGA
(SEQ.I.D.NO:24),
IVS7F 5' ATCTAAGCTTCGTGGAACGAGAGGTGG (SEQ.I.D.NO:25),
IVS7R 5' ATCTAAGCTTGAATTCCTTCCACTCCTGGAGGTT (SEQ.I.D.NO:26),
IVS8F 5' ATCTAAGCTTTGGTGTGTTTGCCCCCT (SEQ.I.D.NO:27),
IVS8R 5' ATCTAAGCTTGAATTCTGCTGGAAGAGAAAACAAA
SEQ.I.D.NO:28),
IVS9F 5' ATCTAAGCTTCTGACCTGCTGGTGAGTAG (SEQ.I.D.NO:29) and
IVS9R 5' ATCTAAGCTTGCCTCTGGCCTACGTCAAGAAAG (SEQ.I.D.NO:30).

PCR fragments were digested with HindIII and cloned into the unique HindIII site of plasmid eIF-40luc, which lies 3' of the transcription initiation site of the eIF4AI gene promoter. Restriction mapping and DNA sequencing was used to identify plasmids containing introns in the correct orientation. Introns 1, 2 and 3 were excised with Hind III, rendered blunt ended by treatment with Klenow DNA polymerase and cloned into the KpnI site of –40eIFluc to give the plasmids IVS1eIF-40luc, IVS2eIF-40luc and IVS3eIF-40luc in which the introns are placed

5' of the eIF4A1 minimal promoter. The plasmid pGL3 Control contains 195bp of the SV40 promoter cloned 5' of the luciferase reporter gene and 244bp of the SV40 enhancer cloned 3' of the luciferase reporter gene (Promega). The plasmid hCMVluc was constructed by cloning the Nru I / Hind III CMV promoter fragment from pcDNA3 (InVitrogen) into the Sma I / Hind III polylinker sites of pGL3 Basic (Promega). The 200bp Herpes Simplex Virus 1 thymidine kinase promoter was PCR amplified and cloned into KpnI/Bg II sites of pGL3 Basic (Promega). The plasmid pcDNA3 Beta gal (14) contains the E.coli lacZ gene cloned into the mammalian expression vector pcDNA3 (InVitrogen). Plasmid DNA for transfection was prepared from 500ml cultures of E.coli grown overnight in LB broth containing 100μg ampicillin (Sigma) using a standard NaOH/SDS lysis protocol and centrifugation in CsCl ethidium bromide gradients (12).

Mammalian cell culture and transient transfection. The murine macrophage cell lines RAW264.7 and P388.D1, the murine B cell line A20 and the human erythroleukaemic cell line K562 and the human prostate cancer cell line, LNCaP were maintained in RPMI 1640 medium (Life Technologies) supplemented with 10% heat inactivated foetal calf serum (FCS) (Sigma, St. Louis, MO), 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 2mM glutamine. CHO K1 and SKOV-3 cells were maintained in Ham's F-12 medium and 293 cells WiDr and COS-1 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS, antibiotics and glutamine. All cells were grown at 37°C in a humidified incubator in 5% CO_2/air mixture.

RAW264.7, P388.D1, A20 and K562 LNCaP and COS-1 cells were transfected by electroporation. Briefly, cells were grown to confluence in T175 flasks, harvested in Phosphate Buffered Saline (PBS), washed once with PBS and resuspended in Optimem 1 serum free medium (Life Technologies) for RAW cells or RPMI 1640 (no FCS). Aliquots of 2 x 10^7 cells (0.5ml) were mixed with 20μg luciferase reporter plasmid DNA and 2μg pcDNA3 β-galactosidase plasmid DNA, added to a 0.4cm electrode gap electroporation cuvette (BioRad, Hercules, CA) and shocked in a BioRad GenePulser (300V, 960μFD) at room temperature. Cells were recovered immediately into 10ml of pre-warmed medium and plated into 9cm diameter tissue culture petri dishes (Nunc).

CHO and 293 cells were grown to 70-80% confluence in 9cm petri dishes, washed twice with Optimem before addition of 5ml of plasmid DNA:cationic lipid complex (5μg DNA: 50μg Lipofectamine (Life Technologies) in Optimem). After 4-6 hours incubation the medium was aspirated, cells were washed twice with PBS and recovered into complete medium for 16 hours before analysis. SKOV-3 and WiDr cells were similarly transfected using 5μg DNA, 50μl lipofectin (Life Technologies)

Reporter gene assays Transfected cells were harvested in 500μl Reporter Lysis Buffer (Promega) and lysed with one cycle of freeze thaw. Cell lysates were assayed for β-galactosidase enzyme activity using the colorimetric substrate chlorphenolred β-D-galactopyranoside (CPRG, Boehringer Mannheim) in a 96 well plate assay in 50mM potassium phosphate buffer (pH7.3) with 2mM $MgCl_2$. Enzyme activity was determined by spectrophotometry at 570nm after 30 minutes incubation at 37°C using dilutions of purified E.coli β-galactosidase enzyme (Sigma) to generate a standard curve. Luciferase enzyme activity was determined using a Luciferase Assay Kit (Promega) and a Berthold Instruments LB9501 luminometer. All luciferase enzyme assays using transfected cell extracts were within the linear range of the assay.

In vivo luciferase assays.

Luciferase reporter plasmids, (50ug), were injected into the quadriceps muscles of C57Bl/6 mice. Muscles were removed after 72 hours and disrupted in 500ul of reporter lysis buffer, (Promega), using an IKA Labortechnic Ultra turrax T8 polytron. Luciferase enzyme activity was determined using a luciferase assay kit, (Promega) and a Dynatech Laboratories ML3000 luminometer. Total protein content was measured in a 96 well format using a Pierce Coomassie Plus Protein Assay Reagent against a standard curve using a Molecular Devices Spectra Max 250 plate reader.

eIF4AI gene organisation

We have shown previously that the macrophage restricted CD68 gene lies 669bp 3' of the gene encoding the ubiquitously expressed translation initiation factor eIF-4AI gene on chromosome 17p13 (13,14). We determined the DNA sequence of 11.9kb of CD68 5' flanking sequence and showed that the human

eIF4A1 gene is 6.2kb in size and contains ten introns shown schematically in Fig.1. The position of the eIF4A1 gene introns and their sizes are listed in Table 1.

Intron	Size	Sequence
Intron 1	1397 bp	AGGATTCCCGgtaagaaagg...aaaccaacagATCCAGAGAC
Intron 2	226 bp	CGTCATCGAGgtgagactgg...catcccctagAGTAACTGGA
Intron 3	440 bp	TGTATCAAGGgtgagacctc...atttgtttagGTTATGATGT
Intron 4	1264 bp	GGCTCAGCAGgtaagagtgg...ctctgctcagATACAGAAGG
Intron 5	363 bp	AGATACCTGTgtgagtaatt...tcttctctagCCCCCAAATA
Intron 6	179 bp	CAACACCCAGgtgagggcag...tttccactagGTAGTTTTGC
Intron 7	81 bp	GGAACGAGAGgtggggccca...caacctccagGAGTGGAAGC
Intron 8	120 bp	ATCCGCCATGgtgtgtttgc...tctcttccagCATGGAGATA
Intron 9	248 bp	TGACCTGCTGgtgagtagag...cttgacgtagGCCAGAGGCA
Intron 10	96 bp	ATATCCACAGgtaagcgtag...tgttttccagAATCGGTCGA

Table 1: eIF4A1 intron sizes are given in base pairs. The exon/ intron boundary sequences are shown with the exon sequences in upper case and the intron sequences in lower case.

The first intron of the eIF4A1 gene contains many CpG dinucleotides and has many features of a methylation free island. An analysis of the first intron DNA sequence revealed the presence of many potential binding sites for mammalian transcription factors. eIF4A1 intron 1 contains three consensus binding sites for Sp1, two C/EBPβ sites, two binding sites for each of the ets factors Elk-1 and c-ets-1 and 8 binding sites for the transcription factor GATA-1. The 440 bp third intron of the eIF4A1 gene contains a 35bp region containing four overlapping consensus binding sites for GATA 1 and three for GATA2 (SEQ.I.E.NO:39).

Comparison of human eIF4A1 promoter sequence with murine eIF4A1 promoter sequence

Comparison of the DNA sequence of the human eIF4A1 gene 5' flanking region with the published sequence of the murine eIF-4AI gene sequence (15) revealed

a 300bp region showing ~85% sequence homology with the mouse eIF-4AI gene proximal promoter. This region contains a canonical TATA box sequence at position –32 relative to the putative transcription start site (16), CCAAT box sequences at positions –65, -141 and –190 and consensus binding sites for the transcription factor Sp1 at positions –51, -74, -177 and -348, (SEQ.I.E.NO:39). Homology with the murine eIF-4AI promoter sequence ends at position –475 but homology with the murine promoter continues at position –1110 and extends for a further 254 bp to the end of the published murine eIF-4AI sequence. The 638 bp region of non-homology with the murine promoter (positions –1109 to –471) contains an extended dinucleotide repeat sequence $(AT)_{38}$, alternating AT repeats have been shown to be present as interspersed repetitive elements in several eukaryotic genomes (17). Analysis of the eIF4A1 5' flanking sequence revealed the presence of AluI interspersed repeats at positions –4588 and –3722 relative to the eIF4A1 transcription start site (Fig.1).

eIF4AI promoter directs expression of luciferase

A 5' deletion series of the eIF4A1 gene promoter was constructed in the luciferase vector pGL3 Basic and plasmid DNAs were introduced into a range of mammalian cell lines by transient transfection. A DNA fragment extending from –526 to + 15 of the eIF4A1 gene directs high-level luciferase reporter gene expression in the epithelial cell lines CHO and 293 and in the murine macrophage cell line RAW 264.7 (Fig. 2A-C). In CHO cells the maximal level of luciferase expression is obtained with plasmid –526eIFluc and the level of expression is similar to that obtained with the SV40 enhancer/promoter vector pGL3 Control (Fig. 2A). In 293 cells the maximal level of luciferase expression is obtained with the –371eIFluc plasmid with the level of luciferase expression being 18 times that obtained with the SV40 enhancer/promoter (Fig. 2B). The –40eIFluc plasmid which contains only the TATA box and the –69eIFluc plasmid which contains a CCAAT box, an Sp1 site and the TATA box give levels of reporter gene expression only slightly higher than that seen with the reporter gene vector alone (pGL3 Basic). The –98eIFluc plasmid which contains an additional 29bp of eIF4A1 promoter sequences contains an AP-2 site and directs high-level gene expression in all three cell lines tested. The –120eIFluc plasmid directs similar levels of reporter gene expression in 293 cells but significantly reduced levels of reporter gene expression are seen in CHO and

RAW 264.7 cells. Addition of eIF4A1 sequences 5' of –120 restores high levels of reporter gene expression.

PCR primers were designed to amplify human eIF4A1 gene sequences 5' of position –1110 that show homology with the murine eIF-4AI promoter. A 1018 bp fragment containing eIF4A1 gene sequences extending from position –2098 to –1080 was cloned in both orientations 5' of the eIF4A1 minimal promoter in the reporter gene vector –40eIFluc to give the plasmids BeIF-40luc and B'eIF-40luc. A 597bp region of the human eIF4A1 promoter that shows no homology with the murine eIF-4AI promoter was cloned into the reporter gene vector –40eIFluc to give the plasmids AeIF-40luc and A'eIF-40luc. In CHO cells the plasmids BeIF-40luc and B'eIF-40luc give levels of luciferase expression only twofold higher than that obtained using the eIF4A minimal promoter (Fig. 3A). In 293 cells the same plasmids enhance expression between 4 and 6-fold (Fig. 3B) while in RAW cells expression is enhanced 5-fold. By contrast the region of non-homology with the murine eIF-4AI promoter present in plasmids AeIF-40luc and A'eIF-40luc enhances expression up to 27-fold in CHO cells, 13-fold in 293 cells and 5-fold in RAW cells (Fig 3).

Analysis of transcriptional regulatory elements in eIF4AI gene introns

eIF4A1 intron 1 contains a methylation free island and potential binding sites for several ubiquitous transcription factors such as Sp1, c-ets1 and Elk-1. Potential transcription factor binding sites are found in other introns of the eIF4A1 gene. We prepared a series of luciferase reporter plasmids in which the first nine introns of the eIF4A1 gene were cloned downstream of an eIF4A1 minimal promoter fragment and tested their activity in transient transfection assays. The data are summarised in Table 2 showing the fold increase in reporter gene activity compared to the eIF4A1 minimal promoter alone (plasmid -40eIFluc).

Plasmid	K562	A20	CHO	293	P388.D1
-40eIF luc	1	1	1	1	1
-40eIF IVS1	260	52.6	29.8	51.6	482
-40eIF IVS2	10.7	7.2	2.2	19.8	5.7
-40eIF IVS3	20	12.6	3.7	26.4	4.3
-40eIF IVS4	0.4	1.9	0.6	0.8	0.6
-40eIF IVS5	7.3	9.5	5.3	14.2	2.9
-40eIF IVS6	10	5.2	4.5	14.3	2.6
-40eIF IVS7	25.3	14.7	5.5	23	6.3
-40eIF IVS8	0.1	0.1	n.d.	n.d.	0.1
-40eIF IVS9	18	8.7	3.3	9.6	2.2

Table 2: Effect of eIF4A1 introns on transcription from an eIF4A1 minimal promoter.

With the exception of introns 4 and 8 seven of nine eIF4AI introns tested were shown to enhance expression when cloned downstream of the eIF4A1 minimal promoter. In all cell lines tested the greatest enhancement of expression is seen when the 1397bp first intron of the eIF4A1 gene is cloned downstream of the eIF4A1 minimal promoter. The magnitude of the observed enhancement varies from 30-fold in CHO cells to 480-fold in the murine macrophage cell line P388.D1. Introns 2, 3, 7 and 9 were able to enhance reporter gene expression more than 15-fold. However, the activity of these introns varied widely between different cell lines, for example, the 83bp eIF4A1 intron 7 enhances luciferase expression 25-fold in K562 cells but only 6-fold in CHO and P388.D1 cells.

To test if intron 1 of the eIF4A1 is able to act as a classical transcriptional enhancer a 1397bp IVS1 fragment was cloned 5' of the eIF4A1 minimal promoter in the plasmid -40eIFluc to give the plasmid IVS1eIF-40luc. Reporter gene plasmids with eIF4A1 placed 5' or 3' of the eIF4A1 minimal promoter were transfected into the murine macrophage cell line RAW 264.7. Placing eIF4A1 IVS1 3' of the eIF4A1 -40 promoter fragment enhances luciferase expression 1700-fold while placing IVS1 5' of the eIF4A1 -40 promoter enhances expression 680-fold (Table 3). Thus, eIF4A1 intron 1 sequences are able to enhance gene expression when placed 5' or 3' of the transcription start site.

	-40eIF luc	-40eIFIVS1	IVS1-40eIF	pGL3 Basic
RLU/ Beta galactosidase (x1000)	60	102000	41000	72
Fold –40eIF luc	1 x	1700 x	683 x	1.2 x

Table 3: The indicated luciferase reporter plasmids (20μg) were electroporated into RAW 264.7 cells with the beta galactosidase reporter plasmid pcDNA3 Beta gal (2μg). Cell lysates were prepared 24 hours post transfection and assayed for luciferase and beta galactosidase enzyme activities. The results shown are representative of two independent experiments.

Sustained high-level reporter gene expression in macrophages using eIF4A1 sequences

In order to develop an expression vector using eIF4A1 sequences we cloned eIF4A1 intron 1 downstream of selected eIF4A1 promoter fragments and compared the level of luciferase expression obtained with eIF4A1 sequences with SV40 and CMV enhancer/promoter sequences cloned into the same reporter gene vector. Addition of the eIF4A1 intron 1 sequence increases reporter gene expression in RAW cells from all the eIF4A1 promoter fragments tested. The level of luciferase activity obtained with the plasmid -271eIFIVS1 is equal to that obtained with the human cytomegalovirus promoter/enhancer in the plasmid hCMVluc and more than three times that obtained with the SV40 enhancer/promoter in the plasmid pGL3 Control (Fig. 4).

The data of Figure 4A were obtained with RAW cells harvested 24 hours post transfection. RAW cells were transfected with CMV and eIF4A1 promoter containing plasmids and a comparison of the level of reporter gene expression over a 96 hour period is shown in Figure 4B. The levels of CMV-driven reporter gene expression in RAW cells declines to less than 10% of initial levels 48 hours post transfection. The level of reporter gene expression obtained in RAW cells with the –271 eIF4A1 promoter fragment and eIF4A1 intron 1 is ten fold greater than that seen using the CMV promoter/enhancer 72 hours after transfection (Figure 4B). The same experiment was performed in non-macrophage cell lines and the data of Figure 4C show a comparison of CMV- and eIF4A1-driven

reporter gene expression in RAW cells. The rapid decline in CMV-driven expression seen in RAW cells is not seen in CHO cells. However the –271eIF-IVS1 plasmid gives sustained high-level reporter gene expression in CHO cells with a five fold increase in luciferase expression between 16 and 48 hours post transfection (Figure 4C).

Expression characteristics in murine macrophages using more than one eIF4A1 intron

The plasmid –40 IVS1 (x1) contains one copy of eIF4A1 gene intron 1 cloned downstream of the –40 eIF4A1 promoter fragment in pGL3Basic and plasmid –40 IVS1 (x2) contains two copies of eIF4A1 gene intron 1 cloned downstream of the –40 eIF4A1 promoter. The plasmid pGL3Control contains the SV40 promoter and enhancer sequences cloned into the luciferase reporter plasmid pGL3Basic.

The indicated plasmid DNAs were introduced into the murine macrophage cell line RAW 264.7, human 293 cells and Chinese Hamster Ovary (CHO) cells along with a beta-galactosidase co-reporter plasmid (pcDNA3 Beta-gal). Cells were harvested 16 hours post transfection and cell lysates assayed for luciferase and beta-galactosidase enzyme activities. Normalised luciferase activities are expressed as fold induction compared to the promoterless vector pGL3Basic (see fig.5). Cloning two copies of eIF4A1 intron 1 downstream of the eIF4A1 –40 promoter gives an increase in reporter gene expression compared to the level obtained using one copy of intron 1 in all three cell lines tested. The additional increase in reporter gene expression is small in RAW cells (10%) but the second copy of eIF4A1 increases gene expression in 293 cells more than five fold.

The eIF4A promoter and intron 1 direct gene expression in vivo in mouse muscle. In vivo eIF4A promoter / intron activity has been determined in mouse muscle by injection of luciferase reporter plasmids, (Fig. 7). The -526 fragment plus intron- 1 shows over three times the activity of the SV40 promoter / enhancer combination in this system.

The eIF4A promoter and intron 1 are active in cancer cell lines. Plasmids where luciferase expression is driven by the eIF4A promoter fragment and intron 1 (IVS-1) regions were introduced into a series of cancer cell lines: LNCaP, COS-1, WiDr and SKOV-3, (Figure 6). Normalised luciferase activities show that the eIF4A promoter and IVS-1 are active in all cell types tested. The -271 and -526 promoter fragments are most active as in other cell types, (see Figs. 2 & 4). For example, the -526 and IVS-1 combination drives almost two fold more luciferase expression than the SV40 promoter / enhancer combination in WiDr, colorectal cancer cells and over 500 fold more expression in LNCaP, prostate cancer cell line.

By combining eIF4A1 promoter sequences with eIF4A1 intron 1 we were able to direct sustained high-level reporter gene expression in macrophages. The levels of luciferase gene expression obtained in murine macrophages using eIF4A1 vectors were three times higher than that seen with the SV40 promoter/enhancer and equal to the level of expression seen using the human CMV promoter/enhancer.

Comparison of transcriptional activity of -271eIF4A and CMV promoters in murine keratinocytes

The inventors have also shown that the eIF4A promoter is more active in murine keratinocytes than the CMV promoter. Again this demonstrates in a cell line other than an antigen presenting cell that the eIf4A promoter is stronger than the CMV promoter. Also murine keratinocytes provide a cell line model for the cell type other than professional APCs that could contribute to the antibody response via the gene gun by expressing antigen *in vivo*. This example shows a sustained high-level reporter gene expression in keratinocytes using eIF4A1 sequences.

Mammalian cell culture

The murine keratinocyte cell line , MK, (Weissman, B.E. and Aaronson, S. A., 1983, Cell **32**, 599-606), was maintained in S-MEM, (Spinner Culture Modified Eagle's Medium) , Life Technologies supplemented with 10% foetal calf serum, Life Technologies, 100 units/ml penicillin, 100ug/ml streptomycin, 2mM

glutamine and 4ng/ml human recombinant EGF, (Sigma). Cells were grown at 37°C in a humidified incubator in 5% CO_2/air mixture.

Transient transfections

MK cells were grown to 80% confluence in 6 well plates, washed twice with 1ml Optimem and transfected with 1ml of plasmid DNA: cationic lipid complex, (5ug DNA: 30ul Transfast ™, Promega), in Optimem. Cells were generally harvested 48 hours post transfection for luciferase assays or at specific time points for a time course of activity.

Luciferase assays

For luciferase assays, transfected cells were harvested in 1ml Passive Lysis Buffer and luciferase activity was determined using a luciferase assay kit, (Promega) and a ML3000 plate luminometer, (Dynatech Laboratories). Total protein content was measured in a 96 well format using Pierce Coomassie Plus Protein Assay Reagent against a standard curve using a Spectra Max 250 plate reader, (Molecular Devices).

Level of transcriptional activity

The level of transcriptional activity of eIF4A1 promoter fragments combined with intron 1, (IVS-1) compared to that of the CMV promoter in murine keratinocytes, was determined. Plasmids containing the relevant promoter / intron combinations driving luciferase expression were transfected into MK cells. Luciferase activity was assayed 48 hrs post-transfection and expressed, (RLU / mg protein), as a percentage of the CMV luc plasmid, (set as 100%). The relative luciferase activity, (Fig 8), indicates that the plasmid –271eIFIVS1 directs about three times as much luciferase expression as the plasmid hCMVluc. Figure 8 represents a mean of 4 separate experiments.

Time course of promoter activity

MK cells were transfected with the plasmid hCMVluc or –271eIF-IVS1luc. Cells were harvested at 22, 31 and 48 hours post transfection and assayed for luciferase actvity and total protein. Relative luciferase activities, (RLU/ mg protein), are given as a percentage of the value from the first time point for each plasmid transfected. Figure 9 is a mean of 2 experiments and shows that the -

271eIF-IVS1 plasmid gives sustained high-level reporter gene expression in MK cells with about a six fold increase in luciferase expression between 22 and 48 hours post transfection. Note that the rapid decline in CMV driven expression seen in RAW cells, (Fig. 4A), is not seen in MK cells.

Efficacy of –271eIF-IVS1 promoter in DNA vaccination

The following example shows that when the eIF4A promoter is driving expression of influenza nucleoprotein (NP) antigen in a particle mediated DNA delivery experiment, it produces as good or a better antibody response than an equivalent plasmid with CMV driven NP expression.

Plasmid construction and DNA preparation

Plasmids used for DNA vaccination experiments are based upon pVAC1, (obtained from Michelle Young, GlaxoWellcome, UK), a modification of the mammalian expression vector, pCI, (Promega), where the multiple cloning site, from EcoR I to Bst Z I, has been replaced by the EMCV IRES sequence flanked 5' by unique Nhe I, Rsr II and Xho I and 3' by unique Pac I, Asc I and Not I restriction enzyme sites. The influenza nucleoprotein, (NP), expression plasmid, pVAC1.PR, (CMV NP-PR), was constructed by ligating PCR amplified cDNA encoding nucleoprotein of influenza A virus strain PR/8/34 from pAR501, (a gift from Dr. D. Kiossis, NIMR, London, UK), into the expression vector pVAC1. To generate the variant of pVAC1.PR containing the –271eIF4A promoter and first intron, (IVS-1), the Msc I / Nhe I fragment containing the CMV promoter and chimeric intron was replaced with a PCR amplified DNA fragment containing the –271eIF4A promoter and IVS-1 flanked by the relevant restriction enzyme sites to create plasmid: -271eIF4AIVS-1NP-PR.

For DNA vaccination experiments, plasmid DNA was propagated in *E.coli* DH5α and prepared using endotoxin-free purification kits, (QIAGEN Ltd., Crawley, UK), and stored at –20°C at approximately 1mg plasmid DNA / ml in 10mM Tris/EDTA buffer.

Cartridge preparation

Preparation of cartridges for particle-mediated DNA delivery (PMDD) using the Accell gene transfer device was as previously described (Eisenbraun et al DNA

and Cell Biology, 1993 Vol 12 No 9 pp 791-797). Plasmid DNA was coated onto 2 μm gold particles (DeGussa Corp., South Plainfield, N.J., USA) and loaded into Tefzel tubing, which was subsequently cut into 1.27 cm lengths to serve as cartridges and stored desiccated at 4°C until use. In a typical vaccination, each cartridge contained 0.5 mg gold coated with between 0.5 – 0.8 μg plasmid DNA.

Animals and immunisations

Female C57Bl/6 mice were immunised between 6-8 week of age. For each immunisation, plasmid DNA was delivered to the shaved target site of abdominal skin by PMDD from two cartridges using the Accell gene transfer device at 500 lb/in^2 (McCabe WO 95/19799), delivering a total of between 1.0 -1.6 μg DNA per immunisation. The DNA vaccination regime included a primary immunisation followed by one boost 6 weeks later.

Blood collection for antibody ELISA assays

Blood samples were collected from the tail vein 1-3 days before immunisation, and at 24 days after primary and 15 days after boost immunisation. Serum was separated and stored at -20°C for subsequent antibody analyses.

Preparation of purified recombinant nucleoprotein (NP) of influenza virus as a glutathione-S-transferase fusion protein for serum antibody ELISA assays

The cDNA for nucleoprotein of influenza A virus strain PR/8/34 was cloned as an *Eco*RI fragment into a modified pUC18 vector. This was then sub-cloned as an *Eco*RI fragment into pGEX-4T-3 (Pharmacia) and DNA sequence analysis confirmed that this construct would express an in-frame fusion with GST.

A one litre culture of *E. coli* DH5α transformed with pGEX flu/NP were grown to a density of OD_{600} ~ 0.8. Fusion protein expression was induced by the addition of isopropyl-b-D-thiogalactopyranoside to a final concentration of 0.1mM and growth continued for a further 3 hours at 37°C. Cells were harvested by centrifugation at 7000 rpm for 7 minutes and the pellet resuspended in 30ml of PBS + 1mM PMSF. Cells were disrupted by sonication at maximum amplitude for 3 x 10s on ice and the insoluble materials were removed by centrifugation at 18,000rpm for 30 minutes. The supernatant was removed and incubated with 1ml of glutathione-sepharose (Pharmacia) for 1h at 4°C with gentle agitation.

The glutathione-sepharose was collected by centrifugation and washed 3 times with 20ml of ice cold PBS. The fusion protein was eluted by incubation of the sepharose with 7x 1ml of elution buffer (10mM reduced glutathione (Sigma) in 50 mM Tris-HCl pH 8) for 10minutes at room temperature. The purified protein was dialysed against 3 litres of PBS to remove the free glutathione and stored at a final concentration of 125 mg/ml in 1ml aliquots at –20°C.

Antibody ELISA assays

Microtitre plates (Nunc Immunoplate F96 maxisorp, Life Technologies) were coated with 10 μg/ml NP by overnight incubation at 4°C and washed 4 times with washing buffer (PBS containing 5 % Tween 20 and 0.1% sodium azide). This was followed by a 1 hour incubation at 20°C with blocking buffer (5% BSA w/v in PBS), and 4 further washes with washing buffer, before the plates were incubated for 4 hours at 20°C with serum samples serially diluted in blocking buffer. After 4 further washes (as above) to remove unbound antibody, plates were incubated for 1 hour with peroxidase conjugated anti-mouse IgG antibodies (Southern Biotechnology, Birmingham, AL, USA) diluted in blocking buffer. The amount of bound antibody was determined after 4 further washes (as above) followed by addition of TMB substrate solution (T-8540, Sigma). After 30 minutes at 20°C and protected from light the reaction was stopped with 1M sulphuric acid and absorbance read at 450 nm. Titres were defined as the highest dilution to reach an OD of 0.2.

Comparison of in vivo effect of -271eIF4A and CMV promoters on IgG antibody response to pVAC1.PR

The NP-specific serum IgG levels were measured before immunisation, and at intervals following the primary and boost immunisations. The construct with the -271eIF4A promoter gave similar responses to those following immunisation with the construct with the CMV promoter (Fig. 10), with equivalent titres for both promoters after the boost immunisation (Fig. 11).

Use of eIF4A regulatory sequence to bias immune response and provoke CTL response

Cartridge preparation

In a typical vaccination, each cartridge contained 0.5 mg gold coated with between either ~0.5, ~0.05 or ~0.005 μg pVAC1.PR plasmid DNA. For the latter two doses, empty vector (pVAC1) was added to provide a total of 0.5 μg DNA/cartridge. All other conditions as for previous Example.

Animals and immunisations

As for previous Example.

Blood sample and tissue collection for ex vivo assays

As for previous Example.

Antibody ELISA assays

As for previous Example except after 4 further washes (as above) to remove unbound antibody, plates were incubated for 1 hour with peroxidase conjugated anti-mouse IgG, IgG1 or IgG2a antibodies (Southern Biotechnology, Birmingham, AL, USA) diluted in blocking buffer.

IFNγ ELISPOT assays

96 well ELISPOT plates (Millipore) were coated with 50 μl rat anti- mouse IFNγ capture antibody (PharMingen) at 15 ug/ml, on the day prior to the assay. This was incubated overnight at 4°C. Non-specific binding was minimised by blocking with 100 μl complete RPMI for at least 1 hour at room temperature.

A single cell suspension of splenocytes was prepared using ground glass microscope slides (super premium microscope slides, 1.0-1.2 mm thick/twin frost (BDH)). Red blood cells were lysed using a standard lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, EDTA). Cells were washed 3 times in complete RPMI, counted and resuspended to give a final density of 4×10^5 cells/well. The following treatments were added in triplicate to give a final volume of 200 μl/well:-

Medium only; IL-2 (50 ng/ml), NP-PR peptide (10 μM) + IL-2, NP-PR peptide only.

The experiment was incubated at 37 C/5% CO2 overnight before detection of IFNγ production. After lysis of cells (H_2O) and washing (PBS), biotin conjugated secondary antibody (biotinylated rat anti-mouse IFNγ, PharMingen) was added at 1 μg/ml and incubated at room temperature for 2 hours. This was followed, after washing with PBS, by incubation with streptavidin-alkaline phosphatase (TCS Biologicals) at 1/1000 of the stock solution for 2 hours at room temperature. Cytokine was finally detected using an alkaline phosphatase substrate kit (Biorad) prepared according to the protocol supplied. The reaction was stopped by washing the plate with H_2O and plates left to dry before enumeration of IFNγ producing cells per 10^6 splenocytes by image analysis.

$Eu3^+$ release assay for detection of CTL activity

These experiments were run in parallel with the IFNγ ELISPOT assays described above in order to confirm functional lytic capacity. A single cell suspension of splenocytes was prepared as previously described (IFNγ ELISPOT). The $Eu3^+$ release assay was carried out after a 5 day period of expansion in vitro. Briefly, effector cells (2×10^7) were restimulated with virus (PR/8/34) pulsed, irradiated (3000 Rads) APC (5×10^6 cells) in a total volume of 10 ml complete RPMI. Following this period of expansion cells were washed and plated out in 96 well tissue culture plate (U-bottomed) to give the required effector:target (E:T) ratios.

Target cells (EL4) were washed once in HEPES buffer before labelling with ice cold labelling buffer (1ml/107 target cells for 40 mins on ice with vigorous shaking every 5 minutes). The reaction was stopped by addition of 9 mls repair buffer with a further 5 minute incubation on ice before cells were washed x 2 with repair buffer and x 2 with complete RPMI. Cells were pulsed with 10 μM cognate peptide for 1 hour at 37°C. (An aliquot was mock pulsed to provide control targets). After washing x 3 with complete RPMI, target cells were plated out at 5×10^3 cells/well, plates centrifuged for 3 minutes at 1000 rpm) and incubated at 37°C for 4 hours. Supernatants were harvested for measurement of $Eu3^+$ release and fluorescence detected by time-delayed fluorimetry in the presence of 200 μl enhancement solution (Wallac).

% specific cytotoxicity was calclulated as:-

(test release – spontaneous release)/(max. release – spontaneous release) x 100%

where test release from target cells was measured in the presence of effectors, spontaneous release, in the absence of effectors, and maximum release in the absence of effectors and with the addition of 10% Triton-X100 as a means of causing cell lysis.

Comparison of in vivo effect of -271EIF4a and CMV promoters on IgG antibody subtype responses to pVAC1.PR

The nucleoprotein PR specific IgG1 and IgG2a responses were determined according to the antibody ELISA assay and were examined 14 days post-boost immunisation. The ELISA assay results are shown in Table 4. The results show that at dosages of 0.005 and 0.05 μg/cartridge the construct containing the -271EIF4a sequence does not give detectable titres of IgG1, whereas high titres of IgG2a were detected (>3.60) at all doses of DNA. An IgG2a response can be correlated to a Th1 cell response and an IgG1 response can be correlated to a Th2 cell response. The results shown in Table 4 thus show a reduced Th2 response and a corresponding bias to a Th1 response when the construct contains the -271EIF4a sequence.

Table 4.

Promoter	***pVAC1.PR dose (μg/cartridge)***	***IgG2a****	***IgG1****
CMV	0.5	4.30	5.39
"	0.05	4.60	4.90
"	0.005	4.60	3.00
-271EIF4a	0.5	4.30	4.95
"	0.05	3.90	none detected
"	0.005	3.60	none detected

*Titres were defined as the highest dilution to give optical density of 0.2 at 450 nm. Results are expressed as the $\log_{10}$ titre for each group.

Comparison of the in vivo effects of the -271EIF4a and CMV promoters on CTL induction

The ELISPOT assay showed that IFNγ positive cells were easily detected following immunisation with the -271EIF4a promoter-driven construct (Figure 12).

A similar pattern of CTL activity was also reflected in Eu3+ lysis assays carried out in parallel (Figure 13).

These assays thus confirm that a cytotoxic T cell lysis (CTL) response is induced after particle mediated DNA delivery where the eIF4A promoter is driving expression.

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